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Inactivation of Paraquat by an Aqueous Extract of *Rehmannia glutinosa**

Jae-Chul Chun,[a]‡ Sung-Eun Kim[a] & Sang-Yong Ma[b]

[a] Department of Agricultural Chemistry, Chonbuk National University, Chonju 561-756, Korea
[b] Department of Life Resources Science, Woosuk University, Wanju 565-800, Korea

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Abstract: *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & Mey. was very tolerant to paraquat (1,1′-dimethyl-4,4′-bipyridinium). The paraquat concentration required to reduce dry weight of *R. glutinosa* by 50% (GR_{50}) was 24 mM, whereas a similar effect was obtained with 0·75 mM in *Zea mays* L. (maize, cv. Dekalb) and *Glycine max* (L.) Merr. (soybean, cv. Kwangkyo). When 1·5 mM paraquat in 10% aqueous extract of *R. glutinosa* was applied to maize and soybean, growth inhibition reached 24% and 7%, respectively, of the untreated control. Decreased activity of paraquat due to the extract also occurred in both leaf discs and chloroplasts of soybean. The total amount of [^{14}C]paraquat absorbed into soybean leaves after 48 h was 34%, but it was reduced to 17% when the extract was added. Translocation of [^{14}C]paraquat was also inhibited in the presence of the extract. In thin-layer chromatography (TLC) analysis using various solvent systems, R_f values of [^{14}C]paraquat with the extract differed from those without the extract. The results suggested that the aqueous extract of *R. glutinosa* contained a substance which could nullify paraquat activity.

Key words: paraquat inactivation, *Rehmannia glutinosa*, tolerance

1 INTRODUCTION

In more than 35 years since the introduction of the bipyridinium herbicide paraquat, this compound has been widely used for total vegetation control in a number of situations. It is a fast-acting, non-selective and non-residual contact herbicide. It is applied to the foliage for the control of annual weeds or applied for selective weed control where contact with the crop is avoided.

Extensive and consecutive use of paraquat has led to incidents of resistance in four grass species and 12 broadleaf weeds worldwide.[1] The paraquat-resistant biotypes are usually tolerant at application rates many times greater than the recommended agricultural dose of the herbicide. Unlike the paraquat-resistant biotypes, Kim and Chun[2] have found that *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & Mey., (Scrophulariaceae; figworts) is naturally resistant to paraquat. This plant has never been exposed to paraquat and there is no susceptible biotype.

In the study of the resistance mechanism of *R. glutinosa* to paraquat, we found that the resistance is related to cell wall binding of paraquat, increased activity of protective enzymes and possibly transformation of paraquat (Chun, J. C., unpublished). TLC analysis using [^{14}C]paraquat showed different R_f values between [^{14}C]paraquat obtained from the paraquat-treated *R. glutinosa* and reference [^{14}C]paraquat. Chun *et al.*[3] reported that there might be a paraquat tolerance-related substance in *R. glutinosa* which confers resistance.

The present study, therefore, was to determine the effect of aqueous extracts from *R. glutinosa* on paraquat

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‡ To whom correspondence should be addressed.

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activity against *Glycine max* (L.) Merr. (soybean, cv. Kwangkyo) and *Zea mays* L. (maize, cv. Dekalb).

2 MATERIALS AND METHODS

2.1 Preparation of the aqueous extract

Rhizomes of *R. glutinosa* were harvested in autumn and dried under sunlight. Two hundred and fifty grams of dried rhizomes were refluxed twice with methanol + water (80 + 20 by volume; 2 litres) for 3 h. After filtering, the filtrates were combined and concentrated to remove methanol using a rotary vacuum evaporator at 40°C. Non-polar fractions in the extract were removed by sequential partitioning in hexane, toluene and dichloromethane. The remaining aqueous fraction was then concentrated to 50 ml.[3]

2.2 Plant materials and treatment

Rhizomes of *R. glutinosa* and seeds of soybean and maize were planted in plastic pots containing a clay loamy soil and grown in a greenhouse at 25°C. When the seedlings reached the three- to five-leaf stage, paraquat (as paraquat dichloride 245 g litre^{-1} SL; Gramoxone®) was applied at rates ranging from 0·75 to 24 mM. Dry weights of the treated plants were measured three days after application (DAA).

The effect of the aqueous extract of *R. glutinosa* on paraquat activity was determined to soybean and maize. A spray solution of paraquat at 1·5 mM was prepared in 10% aqueous extract and applied to the seedlings. At 3 DAA effect of the aqueous extract was compared on the basis of dry weights with the treatment of paraquat without the aqueous extract and the untreated control.

The effect of the aqueous extract applied with paraquat to soybean leaf discs and chloroplasts was also determined. Leaf discs (8 mm diameter) were obtained from the first fully expanded trifoliolate leaves of soybean and 0·1 g of the leaf disc was placed in 4·5 ml paraquat solution in a 15-ml test tube. Paraquat solutions at the rates of 0·1 to 1000 μM were prepared with or without the 10% aqueous extract. The test tubes were shaken for 6 h at 125 μE m^{-2} s^{-1}. After the incubation, the chlorophyll content of the leaf discs was determined spectrophotometrically.[4]

Chloroplasts were isolated using the mechanical method of Mill and Joy.[5] Chloroplasts (22.5 μg Chl ml^{-1}) were incubated in a solution containing sorbitol (0·33 M), Tricine (50 mM; pH 7·6, KOH), magnesium chloride (1 mM), EDTA (2 mM), sodium hydrogen carbonate (10 mM), and a designated concentration of paraquat diluted with or without 10% aqueous extract and illuminated at 125 μE m^{-2} s^{-1}. The experiment was terminated 6 h after treatment (HAT) and the chlorophyll content in chloroplasts was determined spectrophotometrically.[4]

2.3 Uptake and translocation of [^{14}C]paraquat

Soybean seedlings were grown as described above. [^{14}C]Paraquat (10 μl; 0·081 μCi) was spotted onto the first fully expanded trifoliolate leaf. The final concentration of paraquat was made to 3 mM with Gramoxone® after diluting with or without 10% aqueous extract, At 6, 12, 24 and 48 HAT, the leaf surface was washed with distilled water (50 ml) followed by methanol + water (80 + 20 by volume; 50 ml). Amounts of radiolabelled paraquat in the washing solutions were determined in a liquid scintillation counter (Packard A300C, Downers Grove, IL 60515 USA). After washing, the leaf was blotted with paper towels and exposed to a X-ray film for two weeks to compare the translocation patterns of ^{14}C.

2.4 *In-vitro* incubation of [^{14}C]paraquat with the aqueous extract

An aqueous solution of [^{14}C]paraquat containing 0·486 μCi was prepared with or without the 10% aqueous extract of *R. glutinosa.* The solution was shaken for 2 h in the dark at 25°C. After incubation, the solutions were concentrated and analysed by TLC in four different solvent systems. These were: (1) methanol + hydrochloric acid (2 + 3 by volume), (2) 2 M hydrochloric acid + *n*-pentanol (97 + 3 by volume), (3) methanol + benzene + *n*-pentanol + 1 M hydrochloric acid (2 + 1 + 1 + 1, by volume) and (4) 5 M ammonium chloride. TLC plates used were 20 × 20 cm, precoated silica gel 60 F-254 plates (E. Merck, Darmstadt, Germany). Following development, the TLC plates were divided into 1-cm segments from the origin to the solvent front, scraped and radioactivity was quantified by liquid scintillation spectrometry.

3 RESULTS

3.1 Plant response to paraquat

A great difference in paraquat activity was observed between *R. glutinosa* and soybean and maize (Fig. 1). Treatment with 0·75 mM paraquat caused 64% and 59% reduction in the dry weights of soybean and maize, respectively, but had no effect on *R. glutinosa.* No injury to *R. glutinosa* was found at the recommended agricultural dose of 3 mM paraquat. At 24 mM paraquat, the reduction in dry weight of *R. glutinosa* reached 44% of the untreated control. The data clearly show that *R. glutinosa* is very tolerant to paraquat and the level of tolerance is more than 32-fold when compared with soybean and maize.

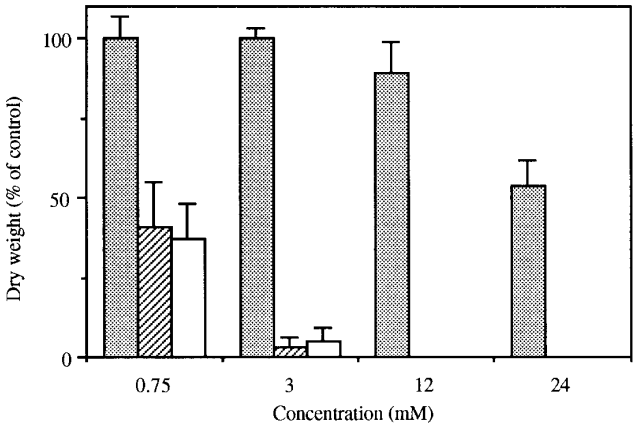

Fig. 1. Effect of paraquat on growth of (▨) *Rehmannia glutinosa*, (▨) maize and (□) soybean. Vertical bars represent standard errors of means.

It has been postulated that paraquat may be adsorbed strongly onto leaf surfaces following its foliar application because of its positive charge.[6] Movement of the bipyridinium herbicide into leaf cells is also likely to be impeded by the thickness of epicuticular waxes found on plant surfaces.[7] However, the epicuticular wax content of *R. glutinosa* is slightly higher than that of soybean, but is lower than that of maize (Chun, J. C., unpublished). Therefore, differences in leaf properties do not appear to be involved in differential tolerance to paraquat among the three species.

3.2 Effect of the aqueous extract on paraquat activity

A decrease in activity of paraquat applied to soybean and maize was observed after addition of the aqueous extract of *R. glutinosa* (Table 1). Application of paraquat alone resulted in almost complete kill, whereas the growth inhibition was greatly reduced when paraquat was applied with the aqueous extract; the visible phytotoxic symptoms included partial chlorosis and marginal necrosis on the leaves. However, the phytotoxic injury did not cause plant death. On the other hand, the aqueous extract alone did not cause any injury. Decreased activity due to the aqueous extract was much greater in soybean than in maize. The difference may be due to differential properties of leaf surfaces such as the amount of epicuticular wax.

TABLE 1
Effect of the Aqueous Extract of *Rehmannia glutinosa* against Paraquat Activity in Soybean and Maize

	Growth inhibition (% of control)[a]		
Plant	*Paraquat*	*Paraquat + aqueous extract*	*Aqueous extract*
Soybean	95	7	0
Maize	92	24	1

[a] The growth response was expressed as a reduction of shoot dry weight caused by the treatments. Mean dry weights of untreated plants were 2·96 (soybean) and 6·36 (maize) g per 10 plants.

For paraquat to be effective following foliar application, it must be absorbed in sufficient amount. Therefore, the decreased activity of paraquat could be due to either reduced absorption or reduced activity in the chloroplasts, or both.

The primary effect of paraquat in light is in chloroplast. Generation of oxygen radicals in illuminated photosynthetic tissues treated with paraquat results in simultaneous peroxidation of lipid and oxidation of chlorophyll.[8] Chlorosis as determined by chlorophyll content is a rapid and accurate indication of paraquat activity in the treated leaves. A reduction in the level of paraquat-induced chlorophyll degradation was found when the aqueous extract was added (Fig. 2). In leaf discs of soybean, the concentration of paraquat required to reduce the chlorophyll content by 50% (CHL_{50}) was 33 μM, whereas the CHL_{50} increased to 90 μM when the aqueous extract was added. A similar increase in CHL_{50} was observed in the chloroplasts. The CHL_{50} values for paraquat treatment without and with the

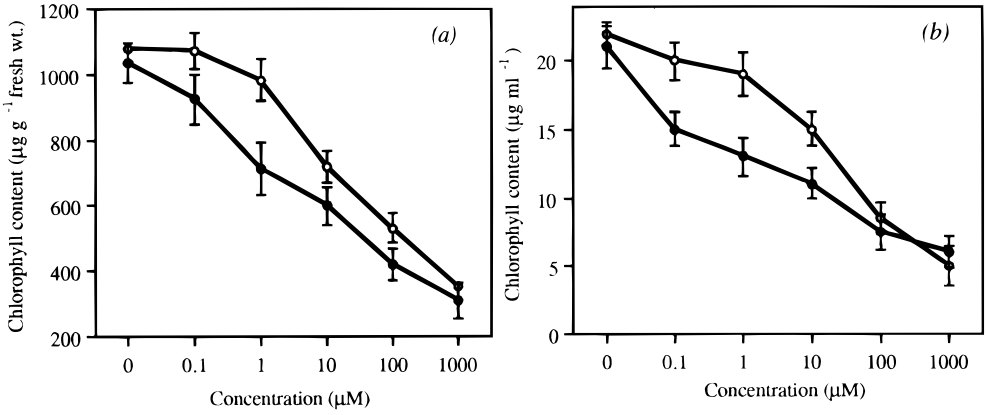

Fig. 2. Effect of the aqueous extract of *Rehmannia glutinosa* against paraquat activity in (a) leaf discs and (b) chloroplasts of soybean. (—●—): paraquat alone; (—○—): paraquat + aqueous extract of *R. glutinosa*. Vertical bars represent standard errors of means.

TABLE 2
Effect of the Aqueous Extract of *Rehmannia glutinosa* on Absorption of [^{14}C]paraquat into Trifoliolate Leaves of Soybean

Treatment	*Exposure time (h)*	*Radioactivity in washes (% of applied)*[a]		*Absorption (% of applied)*[a]
		H_2O	CH_3OH	
[^{14}C]Paraquat	6	69 b	2 b	29 a
	12	67 b	1 b	32 a
	24	65 b	<1 b	34 a
	48	65 b	<1 b	34 a
[^{14}C]Paraquat + Aqueous extract	6	81 a	5 a	14 b
	12	79 a	4 a	17 b
	24	81 a	2 ab	17 b
	48	78 a	4 a	18 b

[a] Means within columns with same letters are not significantly different at the 5% level by the LSD test.

aqueous extract were 8 μM and 43 μM, respectively. The effect of the aqueous extract in both leaf discs and chloroplasts was obvious at concentrations of paraquat lower than 10 μM. The results obtained suggest that there is a direct interaction between the aqueous extract and paraquat.

3.3 Effect of the aqueous extract on absorption and translocation of [^{14}C]paraquat

The data in Table 2 show that about one-third of the applied [^{14}C]paraquat was rapidly absorbed within 6 HAT. There was no significant increase in absorption of [^{14}C]paraquat after 6 HAT. When [^{14}C]paraquat was applied with the aqueous extract, however, a significant reduction in absorption of radioactivity was observed. In contrast, there was a significant increase in radioactivity in washes of water and methanol.

The effect of the aqueous extract on absorption and translocation of [^{14}C]paraquat was also confirmed by autoradiography. The autoradiography shown on the top of Fig. 3 illustrates greater mobility of the absorbed radioactivity into the main veins of the trifoliolate leaves of soybean over time. In contrast, the radioactivity applied with the aqueous extract shown on the bottom autoradiograph did not move and remained at the treated site. These results indicate that the aqueous extract impedes both absorption and translocation of paraquat.

3.4 TLC analysis of [^{14}C]paraquat incubated *in vitro* with the aqueous extract

Using four different TLC systems, different major bands of radioactivity appeared in lanes of TLC plates spotted with standard [^{14}C]paraquat and [^{14}C]paraquat + aqueous extract (Table 3). In solvent system 1, most of the standard [^{14}C]paraquat was observed at R_f 0·4–0·6. Other less intense bands of the radioactivity appeared with different R_f values. These R_f values are similar to those reported by Kim and Hatzios.[9] In other solvent systems, the major bands had different R_f

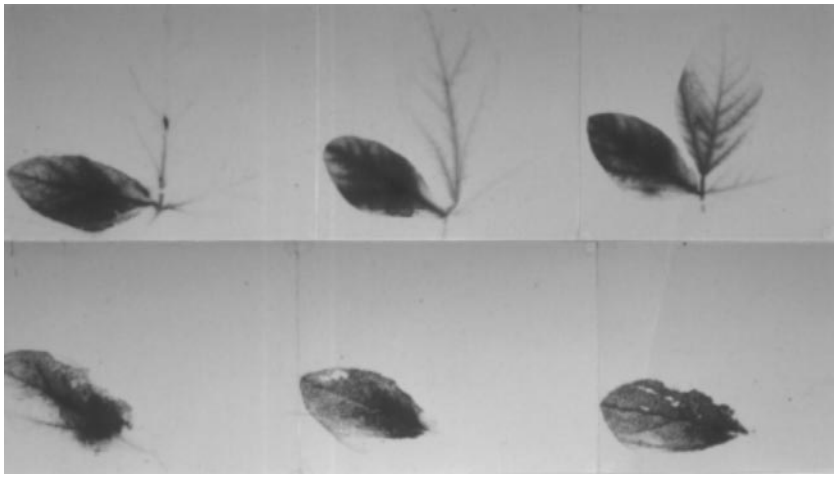

Fig. 3. Autoradiographs showing the distribution of radioactivity in intact trifoliolate leaves of soybean after (left) 6, (centre) 12 and (right) 24 h of [^{14}C]paraquat treatment. (top) [^{14}C]paraquat alone, (bottom) [^{14}C]paraquat + aqueous extract of *Rehmannia glutinosa.*

TABLE 3
TLC Analysis of [^{14}C]paraquat incubated *in-vitro* with and without the Aqueous Extract of *Rehmannia glutinosa*

Incubation solution	*Solvent system*[a]	*Distribution (R_f) of radioactivity (% of recovered)*				
		0·0–0·2	*0·2–0·4*	*0·4–0·6*	*0·6–0·8*	*0·8–1·0*
[^{14}C]Paraquat	1	3	18	78	<1	<1
	2	4	12	83	<1	<1
	3	95	3	<1	1	<1
	4	18	72	7	2	1
[^{14}C]Paraquat + aqueous extract	1	55	39	4	<1	<1
	2	47	36	16	<1	<1
	3	23	64	11	<1	1
	4	61	23	12	3	1

[a] Solvent system: (1) methanol + hydrochloric acid (2 + 3 by volume); (2) 2 M hydrochloric acid + *n*-pentanol (97 + 3 by volume); (3) methanol + benzene + *n*-pentanol + 1 M hydrochloric acid (2 + 1 + 1 + 1 by volume); (4) 5 M ammonium chloride.

values. Unlike the R_f values of standard [^{14}C]paraquat, very different distribution patterns of radioactivity were observed in the different solvent systems used, after incubation with the aqueous extract. These data suggest that paraquat has been altered to another form by the aqueous extract.

4 DISCUSSION

The major goal of the present study was to determine the effect of the aqueous extract obtained from the paraquat-resistant plant, *R. glutinosa*, on paraquat activity. The data presented herein are consistent with a previous report which indicates that *R. glutinosa* possesses natural resistance to paraquat.[2] Data presented in this report also show that paraquat can be inactivated by an aqueous extract obtained from *R. glutinosa*. When paraquat was applied with the aqueous extract to susceptible plants, the activity of the paraquat decreased at the whole plant level or in the leaf discs as well as at the site of paraquat action, the chloroplast. This observation implies that the action of the aqueous extract on paraquat activity is not based on the target plant, but originates from a direct interaction between the two components.

The fact that there is a direct interaction between paraquat and the aqueous extract could be demonstrated from absorption and translocation as well as *in-vitro* incubation studies. [^{14}C]Paraquat applied with the aqueous extract was less able to enter the leaf cuticle, resulting in a decrease in absorbed radioactivity as compared with that when [^{14}C]paraquat was applied alone. Moreover, translocation of the absorbed radioactivity was also impeded. The restricted absorption and translocation is due possibly to transformation of paraquat. Occurrence of paraquat transformation in the aqueous extract was also confirmed *in vitro* by TLC analysis. Major bands of the radioactivity recovered after incubation with the aqueous extract did not correspond to those of standard [^{14}C]paraquat in any of the solvent systems used. These results suggest that paraquat may have been chemically transformed or physically bound to a substance present in the aqueous extract of *R. glutinosa*. In this case biotransformation of paraquat is possibly excluded, since the aqueous extract obtained by refluxing at above 70°C may not contain any biological components.

Future work should address the identification of the substance present in the aqueous extract causing tolerance and a possible role of this substance in the mechanism of tolerance of *R. glutinosa* to paraquat. Since *R. glutinosa* shows a natural resistance to paraquat and the aqueous extract contains a substance which nullifies paraquat activity, it can be postulated that the resistance mechanism of *R. glutinosa* to paraquat may be based in part on presence of a tolerance-related substance.

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